Practical Kinetics II: Quantitation of Procaine Stability by TLC

H. V. MAULDING ˣ and A. F. MICHAELIS

Abstract □ This work illustrates further application of TLC as a tool in kinetic investigations of organic molecules. The procedure consists of: (*a*) streaking or spotting a known volume of reaction solution on thin-layer plates, (*b*) developing the plates, (*c*) eluting the compound under study from the adsorbent, and (*d*) assaying the material in solution. Procaine was examined regarding its hydrolysis at above ambient temperatures. The reaction was monitored spectrally and by the TLC technique. Velocity constants derived by the two methods were similar in value. Rate constants obtained from procaine samples eluted from silica gel plates following development were in good agreement with those previously reported in the literature.

Keyphrases □ Procaine—quantitation of stability by TLC, rate constants □ Hydrolysis—quantitation of procaine stability by TLC, rate constants □ Stability, procaine—TLC determination □ TLC—determination, procaine stability

Further utilization of quantitative TLC as a technique for rapid and accurate kinetic screening of solutions is reported. The method is broadened to include basic and cationic compounds whereas previously acidic or anionic substances, the barbiturates, were surveyed.

The same general approach is extended to a basic compound, procaine, by means of alterations of the elution solvents. Procaine was used as a prototype due to its medicinal importance as well as the relatively large amount of data regarding the rate constants of its decomposition available (1–3).

This amino ester, pKa_1 8.95, is hydrolyzed in solution, with consequent production of *p*-aminobenzoic acid and diethylaminoethanol. The examined solutions were an order of magnitude below those normally employed medicinally. The commercial article could be analyzed following a dilution.

EXPERIMENTAL

TLC—Procaine hydrochloride (mp 152–155°) was found chromatographically [methanol–ammonia (99:1)] to contain only traces of *p*-aminobenzoic acid. This substance was used without further purification. The methanol–ammonia solvent system was used to develop kinetic samples with a running distance of 15 cm on silica gel fluorescent plates[1]. Procaine, R_f 0.7–0.8, and *p*-aminobenzoic acid, R_f 1.0, were visualized under short wavelength UV light (255 nm). This system is an adaptation of a previously reported (4) solvent system.

Kinetic Studies—A solution of procaine hydrochloride (2 mg/ml) was prepared in the appropriate buffer solution. The samples were placed in constant-temperature baths (±0.1°), and aliquots were periodically withdrawn by pipet as the reaction progressed. A 0.25-ml[2] portion of the reaction solution was streaked[3] on full-sized (20 × 20 cm) silica gel fluorescent plates[1], with two samples placed one on each half of a manually scored plate. Forced air drying was used to confine the bandwidth as the plate was streaked.

The plate was developed in methanol–ammonia (99:1), and the band of silica gel containing the starting material was marked under UV light. The silica gel containing procaine was removed by scraping with a microspatula and collected. This material was extracted with 10 ml of methanol followed by pH 6 phosphate buffer to make 50 ml. The solid particles of silica gel were removed by filtration through a sintered-glass filter followed by analysis of the solution.

Analysis of Solutions of Procaine from TLC Plates—The methanol–pH 6 buffer from the preceding elution of the thin-layer plates was read on a UV recording spectrophotometer[4]. The absorbance was read at 289 nm (±1) against the appropriate blank.

UV Analysis—Samples were taken concomitantly with those used for the TLC assay. The aliquots were diluted to volume with the appropriate buffer, with the absorbance values taken as a function of time at 289 nm (±1). The UV analyses of intact procaine were carried out directly, without need for separation, at 289 nm following pH adjustment to 9.5. This was possible due to the disparity in molar absorptivities of procaine and *p*-aminobenzoate anion, with values of 7.5 and 17.5 × 10^3 liters/mole cm, respectively (1).

The equation employed was:

$$\log (A_t - A_\infty) = \log (A_0 - A_\infty) - kt/2.303 \quad \text{(Eq. 1)}$$

where A_t is absorbance at time t, A_∞ is the absorbance at infinite time (*i.e.*, *p*-aminobenzoate ion), and A_0 is the initial absorbance or that of procaine at time = 0.

Buffer Solutions and pH—Ammonia buffers were prepared following the general directions of Higuchi *et al.* (1), and borate buffers were made as previously specified (5) (Table I).

The pH measurements were carried out on a pH meter[5] standardized at the specified temperatures by borate and phthalate solutions (6). The pH of the reaction solutions was determined before, during, and following each kinetic run.

RESULTS AND DISCUSSION

This work comprises an expansion of the TLC method as a means for rapidly determining the kinetic parameters of solutions. The technique has been found suitable for formulation screening as well as for classical kinetic investigations. The process depends upon separation of the starting material (usually 0.1–0.5 mg) from products on thin-layer plates, which is possible in theory and generally in practice.

Barbituric acids and their anionic forms were previously shown to be fitted to analysis in this manner. Ramification of the technique is presented to include basic compounds and their cationic forms. The amino ester, procaine, was readily amenable to this treatment. This compound was selected due to the large amount of data regarding the velocity constants and other parameters of its hydrolysis already available (1, 2).

The cleavage of the ester linkage was monitored spectrophotometrically. Procaine was separated from *p*-aminobenzoic acid by TLC with the starting material being eluted from the plates and then read in the UV at 289 nm (±1).

The expression:

$$\log A_t = \log A_0 - kt/2.303 \quad \text{(Eq. 2)}$$

[1] Silica gel 60 F-254, 0.25-mm plates, EM Labs. Inc., Elmsford, N.Y.
[2] Using a 0.5-ml syringe fitted for sieve collar attachment, Hamilton Co., Inc., Whittier, Calif.; Applied Science Catalog No. 17710.
[3] Streaker apparatus made by Applied Science Labs. Inc., State College, Pa.; Applied Science Catalog No. 17700.
[4] Perkin-Elmer 202.
[5] Metrohm.

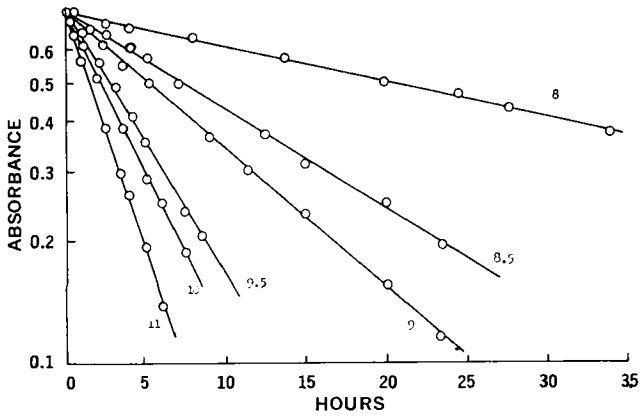

Figure 1—*Decomposition of procaine (concentration of 2 mg/ml at 40°) in aqueous solution as a function of time. Various pH values are indicated on the graph. Analyses were by quantitative TLC.*

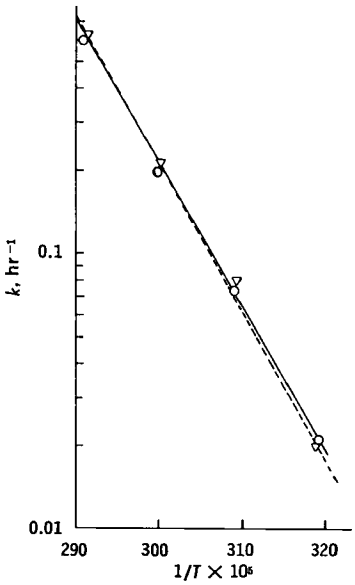

Figure 3—*Temperature dependency for hydrolysis of cationic procaine in pH 8 buffer at 40°. Key: ---▽---, rate constants evaluated by TLC procedure; and —○—, rate constants determined by spectrophotometric analysis.*

was utilized for this pure material, where A_t and A_0 represent absorbances at times t and zero. The term k is the observed or pseudo-first-order rate constant, with t being the time.

The procaine in solution was followed using the equation:

$$\log (A_t - A_\infty) = \log (A_0 - A_\infty) - kt/2.303 \quad \text{(Eq. 3)}$$

where the terms are the same as in Eq. 2 except A_∞, which is the absorbance at infinite time.

Table I lists the observed first-order velocity constants derived from the thin-layer method (Eq. 2) along with those obtained simultaneously by the spectrophotometric procedure (Eq. 3). These are compared to those previously reported by other workers (1, 2).

Kinetic runs were carried out in duplicate for each pH and temperature investigated. The method is quick and applicable from operator to operator, usually in ±10% limits. Once, unacceptable results were found by both the thin-layer and spectroscopic methods. The plots of log concentration, *i.e.*, absorbance, *versus* time resulted in a curved line in both cases. Something apparently was wrong with the solution employed. This was shown to be a loss of ammonia from unsealed flasks containing ammonia–hydrochloric acid buffer with subsequent change (lowering) of pH.

At least seven data points were taken for each run listed in Table I. Table II illustrates five typical runs by the same operator at pH 9.5 and 40° and gives an idea of the reproducibility of the technique.

Problems were encountered with the ammonia buffers. The pH values often changed by as much as one unit at higher pH's during the run. The phenomenon is well known and was previously reported (1). For this reason, borate buffers were found to be much more acceptable in the pH 8–11 region (5). None of the borate buffers exhibited this tendency to change pH during the reaction.

Most runs were carried out for a minimum of two half-lives. Below this point the analytical points tended to show more scatter as assayed by both methods.

The thin-layer separation of reactant and products is a prime

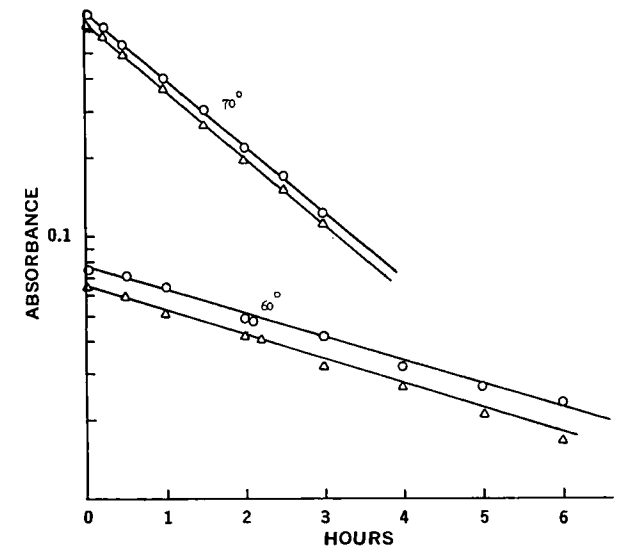

Figure 2—*Comparison of TLC and spectrophotometric results of hydrolysis of 2 mg/ml procaine at pH 8 at 60 and 70°. Absorbance is plotted* versus *time. Key: ○, spectrophotometric absorbances; and △, TLC absorbances. Two "semilog" scales were utilized for convenience in visualization.*

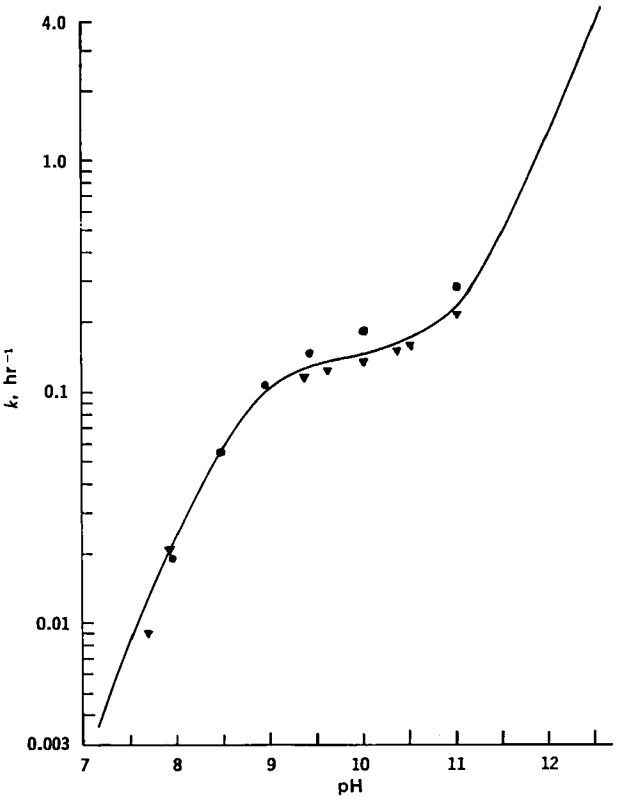

Figure 4—*Log k–pH profile of procaine at 40°. Solid curve represents a plot of the results of Higuchi* et al. *(1). Key: ●, data obtained by Siegel* et al. *(2); and ▼, results obtained from TLC (Table I). Values were determined in this work in borate buffer (Table I).*

Table I—Observed First-Order Rate Constants[a] (k, hr^{-1}) for Hydrolysis of Procaine (2 mg/ml) in Aqueous Solution at Various Hydrogen-Ion Concentrations and Temperatures

Buffer[b]	pH[c]	Temperature	Velocity Constants[d] TLC	Velocity Constants[d] Spectrophotometric	Ref. 1	Ref. 2
Ammonia–hydrochloric acid	7.6	40°	0.007	0.006	0.009	—
Ammonia–hydrochloric acid	8.0	40°	0.018	0.017	0.021	0.018
Borate	8.0	40°	0.021	0.024	(0.021)[e]	—
Borate	8.0	50°	0.079	0.075	(0.073)[e]	—
Borate	8.0	60°	0.21	0.22	(0.21)[e]	—
Borate	8.0	70°	0.63	0.61	(0.64)[e]	—
Borate	8.0	40°	0.020	0.019	(0.021)[e]	—
Ammonia–hydrochloric acid	8.5	40°	0.042[f]	0.046[f]	0.058	0.053
Ammonia–hydrochloric acid	9.0	40°	0.080[f]	0.084[f]	0.105	0.116
Borate	9.0	40°	0.101	0.099	(0.105)[e]	—
Ammonia–hydrochloric acid	9.5	40°	0.09[f]	0.10[f]	0.13	0.15
Borate	9.5	40°	0.12	0.12	(0.13)[e]	—
Ammonia–hydrochloric acid	10.0	40°	0.12[f]	0.12[f]	0.15	0.40
Borate	10.0	40°	0.14	0.15	(0.14)[e]	—
Borate	10.5	40°	0.16	0.17	(0.17)[e]	—
Ammonia–hydrochloric acid	11.0	40°	0.15[f]	0.16[f]	0.21	0.31
Borate	11.0	40°	0.19	0.18	(0.21)[e]	—

[a] Determined following elution of TLC plates with methanol–pH 6 phosphate buffer read at 289 (±1) nm. [b] Borate buffer prepared by adding boric acid to 8 g of sodium chloride in 500 ml of water. When proper pH is attained, add water to 1 liter. Ammonia buffers prepared as in Ref. 1. [c] The pH was measured before, during, and after the kinetic run. [d] Velocity constants taken from same aliquot for TLC and spectrophotometric analysis. [e] Carried out in ammonia–hydrochloric acid buffer in Ref. 1. In this work it was done as indicated in borate. [f] Values low, probably due to pH drop during the reaction of unsealed reaction vessels.

requisite of the procedure. It was effected using the system of methanol–ammonia (99:1) in a modification of a previously reported development solution (4). This separation eliminates the need for development of a differential analytical method, which can pose problems when products interfere, *etc.* The spectra obtained from the eluate should be that of the pure compound, procaine, as was found to be the case.

Procaine was readily removed from the adsorbent, fluorescent silica gel, by means of methanol followed by pH 6 buffer. Methanol and methanol–0.1 *N* HCl or acidic buffers have been generally acceptable for the several amines studied to date.

Care and precaution must be taken when deciding on the solvent for elution of the adsorbent because first trials may not lead to proper extraction. It is best if one spends a day or two removing known amounts of material from the plates and thereby selects the best solvent or solvent system prior to initiation of kinetic studies. Choice of a suitable extraction solvent is sometimes tricky but is possible with all compounds studied to date.

Figure 1 gives typical plots of log absorbance *versus* time derived from various pH values at 40° as done by quantitative TLC.

Figure 2 shows a comparison of data derived from spectrophotometric and thin-layer procedures at different hydrogen-ion concentrations. Close correlation between the rate constants from the two methods is obvious.

An estimate of the apparent energy of activation for the reaction of protonated procaine and hydroxide ion, pH 8, is given in Fig. 3. Utilizing the Arrhenius relationship:

$$\log k = \log P - Ea/2.303RT \qquad \text{(Eq. 4)}$$

where k is the observed first-order rate constant. The plot of log k against $1/T°$ (absolute) gives a straight line, the slope of which is equal to $Ea/2.303R$. The apparent energy of activation, Ea, was 25.6 kcal/mole for the TLC procedure compared to 23.8 kcal/mole from the solution directly (Fig. 3). Higuchi *et al.* (1) derived 24.0 kcal/mole for the same reaction.

Table II—Reaction Velocity Constants for Decomposition of Procaine in pH 9.5 Borate Buffer[a] at 40°

Run	k, hr^{-1}
1	0.11
2	0.13
3	0.12
4	0.12
5	0.13
Average[b]	0.12[c]

[a] Ammonia–hydrochloric acid buffers give somewhat lower values due to change of pH with time. [b] Average of spectrophotometric runs was 0.12. Value from Ref. 1 was 0.13 using ammonia–hydrochloric acid buffer. [c] Rate constants were measured following elution of plates and read at 289 (±1) nm in methanol–pH 6 phosphate buffer. See *Experimental*.

Figure 4 represents a comparison of data obtained from the literature with those in Table I. The continuous solid line records a replot of the results of Higuchi *et al.* (1). This is a graph of the relationship:

$$\log k_{obs} = k_1[OH^-]_{fBH^+} + k_2[OH^-]_{fB} \qquad \text{(Eq. 5)}$$

where f_{BH+} and f_B are fractions ionized and nonionized, respectively; and k_1 and k_2 are rate constants with $pKa_1 \approx 9$.

Various data points for the log k–pH profile are seen along with those obtained in this study. Reasonable correlation may be seen between the three sets of results, with a slight possible error resultant from estimations necessarily made in replotting.

No series defects in the total system were noted during the investigation. One spurious set of data was obtained by both methods examined. One unanswered question was the fact that initial assays by both methods were not always the same absolute values as they should have been assuming homogeneous solution and negligible breakdown. Figure 2 illustrates this matter. No effect on the latter assays was noted; the phenomenon is unexplained, with no aberration in the velocity constants being brought about by this incongruity. The reason for this is that as long as a constant value is streaked, the relative results are the same if the solution is homogeneous.

The TLC procedure is an inexpensive and simple method. No elegant equipment is needed as may be the case with gas or liquid chromatography. The total cost including the plate streaker is less than $500.00. It also possesses the potentiality of being applicable to most compounds.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 23, 1974, from the *Pharmacy and Analytical Research Department, Sandoz-Wander, Inc., East Hanover, NJ 07936*

Accepted for publication July 2, 1974.

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Practical Kinetics III: Benzodiazepine Hydrolysis

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Abstract □ The velocity constants for chlordiazepoxide hydrolysis were measured by independent techniques. A quantitative TLC kinetic procedure is compared with an extractive method. The data derived from both processes are in approximate agreement, further exemplifying the feasibility of TLC for rapid stability evaluation of liquid formulations as well as solution kinetic studies. In the extractive procedure, benzodiazepine–substrate was separated from the lactam product by methylene chloride extraction of acidic aqueous solution. The TLC procedure consisted of separation on silica gel plates followed by elution and subsequent analysis. The log k–pH relationship for the hydrolysis representing water addition coupled with expulsion of methylamine is presented. This function is characterized by water and hydroxide-ion attack on monoprotic species along with specific hydrogen-ion catalysis at higher hydronium-ion concentrations, and the rate law for the decomposition of chlordiazepoxide is given. Through several half-times (pH 0.15–11.5, 79.5°), this hydrolytic reaction generating lactam predominated; however, more benzophenone was formed as the pH decreased. Velocity constants were invariant over a 200-fold concentration range. The subsequent acid-facilitated cleavage of lactam to benzophenone was not further investigated. Both general acid catalysis and general base catalysis were evidenced, with borate, acetate, formate, and phosphate buffers accelerating the conversion of chlordiazepoxide to lactam. At pH values below neutrality, nonlinear dependency of the rate constant on buffer concentration was observed. This finding may be explained by a change in the rate-determining step as buffer concentration varied.

Keyphrases □ Benzodiazepines—hydrolysis of chlordiazepoxide, velocity constants, conversion to lactam, TLC and extraction procedures compared □ Chlordiazepoxide—hydrolysis, velocity constants, conversion to lactam, TLC and extraction procedures compared □ Hydrolysis—benzodiazepines, velocity constants for chlordiazepoxide hydrolysis, TLC and extraction procedures compared □ Kinetics—benzodiazepine hydrolysis

This investigation was undertaken to study the kinetics of chlordiazepoxide hydrolysis as well as to compare pertinent rate data concerning breakdown of the compound using both classical and quantitative TLC analytical techniques.

TLC techniques have been utilized in these laboratories for rapid determination of solution stability of diverse classes of medicinal agents. TLC is applicable when separations of reactant–product(s) are available, coupled with satisfactory analytical procedures for eluted compounds.

The benzodiazepines were of interest because they represent prototypes giving mutual substrate–product interference in the UV region, limiting straight UV spectroscopy in their study (1). Therefore, a kinetic study of the hydrolysis was instituted using both quantitative TLC and spectrophotometric (following separation of reactants from products) methods. The aim, besides delineation of the kinetic profile of chlordiazepoxide hydrolysis, was to compare the agreement between techniques.

In the spectrophotometric procedure, the lactam formed by hydrolysis was readily removed from acidic solutions of the parent, chlordiazepoxide, by partitioning into methylene chloride, allowing a comparative means for determination of velocity constants.

EXPERIMENTAL

Materials—Glass-distilled water was used in all experiments. Reagent grade inorganic salts were employed without further purification.

Chlordiazepoxide reference standard[1], 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide reference standard[1], and 2-amino-5-chlorobenzophenone[1,2] were used.

Kinetic Measurements—Stock Solution 1A was prepared containing 0.90 mg/ml chlordiazepoxide. Two milliliters of this solution was pipetted into 200-ml flasks containing 198 ml of appropriate buffer solution previously equilibrated at the specified temperature, giving a final concentration of 2.98×10^{-5} M. Ten-milliliter samples were periodically withdrawn by pipet and then acidified below pH 1.1 by dropwise addition of concentrated hydrochloric acid.

The aqueous acidic solution was extracted with 3 × 10-ml portions of reagent methylene chloride (methylene chloride was discarded) in a separator, and the aqueous layer was read in the UV at 244 (or 305) nm. Residual absorbances were generally less than 5% of the initial readings and were subtracted for calculation of velocity constants. First-order rate constants were readily evaluated from:

$$k_{obs} = 0.693/t \qquad \text{(Eq. 1)}$$

The observed rate constants derived in this manner were generally reproducible within ±10%.

Stock Solution 2A of chlordiazepoxide (1–2 mg/ml) in appropriate buffer solutions was prepared and placed in constant-temperature baths, 79.5°; aliquots were withdrawn periodically by pipet. A 0.25-ml sample was streaked[3] by means of a streaker apparatus[4] on 20 × 20-cm, 250-μm, silica gel fluorescent plates[5]. One sample was placed on each half of the scored plate with drying by warm air. The plate was developed in *fresh* benzene–dioxane–ethanol–ammonia (50:44:5:1), and the silica gel band corresponding to the

[1] NF reference standards.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Applied Science Laboratories Catalog No. 17710, Hamilton syringe, Hamilton Co., Whittier, Calif.
[4] Catalog No. 1700, Applied Science Laboratories, Inc., State College, Pa.
[5] Analtech, Newark, Del.